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ORIGINAL ARTICLE

Expression patterns of natriuretic peptides in pre-hibernating and hibernating Anatolian ground squirrel (*Spermophilus xanthoprymnus*) kidney

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Abstract

Hibernation is characterized by marked suppression of renal function. Natriuretic peptides (NPs) are involved in the regulation of renal function. However, the role of NPs in the renal function during hibernation remains unclear. We aimed to investigate the distribution patterns of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) in Anatolian ground squirrel (*Spermophilus xanthoprymnus*) kidneys during pre-hibernation and hibernation periods. Cortical proximal tubules showed weak ANP immunoreactivity, with moderate staining on the brush border during the pre-hibernation period. In the hibernation period, moderate ANP immunoreactivity was seen in cortical proximal tubules, with very weak reaction in hibernating cortical distal tubules, medullary proximal and collecting tubules. Cortical proximal and distal tubules of both periods had strong and weak BNP immunoreactivity, respectively. Medullary proximal, distal and Henle's loop segments showed very weak BNP immunoreactivity during pre-hibernation. Medullary distal, proximal and collecting tubules and Henle's loop segments had moderate staining during hibernation. In both periods, cortical proximal tubules displayed strong immunoreactivity to CNP. Distal tubules had moderate CNP staining during pre-hibernation, albeit weak staining during hibernation. Medullary proximal tubules exhibited moderate to strong immunoreactivity during pre-hibernation. Medullary distal and proximal tubules had weak and moderate CNP staining, respectively, during pre-hibernation. In both periods, Henle's loop segments displayed moderate CNP immunoreactivity. Glomeruli had similar weak ANP, BNP and CNP staining in both periods. These results suggest that heterothermic conditions differently affected the expression of NPs in the squirrel kidney. This different expression of NPs may contribute to the renal adaptation during hibernation.

Keywords Natriuretic peptides · Hibernation · Anatolian ground squirrel · *Spermophilus xanthoprymnus* · Kidney

Introduction

Mammalian hibernators, including the Anatolian ground squirrels examined here, enter heterothermic conditions that become less energetically cost. The heterothermic behaviors observed in those animals oscillate repeatedly between dramatically distinct states of torpor and arousal (Jani et al. 2012). The body temperature hovers around ambient temperature during torpor. Hibernators slow dramatically their physiological activities down compared to those at euthermic levels. They reduce their breathing rate from a euthermic level of 100–200 breaths per minute to a hypothermic level of 4–6 breaths per minute and their heart rate from a euthermic level of 200–300 beats per minute to a hypothermic level of 3–5 beats per minute, for example (Carey et al. 2003). However, many physiological functions, such as metabolic, heart, and respiratory rates, return to euthermic levels during arousal, and the body temperature increases to near 37 °C with rewarming process employing exactly endogenous mechanisms of heat production (Staples 2014). The ability of the hibernator to continue consecutive cycles of prolonged periods of torpor

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punctuated by rapid reperfusion with subsequent rewarming serves a natural model of successful organ preservation for clinical conditions such as renal transplantation throughout cold storage followed by warm reperfusion (Zancanaro et al. 1999; Carey et al. 2003; Lindell et al. 2005; Jani et al. 2011).

Renal function is considered to effectively cease during hibernation, as such in kidneys experiencing cold storage during organ preservation (Hong 1957; Tempel et al. 1977). Regional blood flow and arterial pressure within the kidney is greatly reduced, which in turn precludes glomerular filtration (Bullard and Fijnkhouser 1962; Tempel et al. 1977). Urine formation comes to a standstill during torpor and at the beginning of arousal (Moy 1971). The lowered arterial pressure followed by loss of glomerular filtration in the hypothermic, torpid condition is restored during every natural arousal period that terminates the hibernation. Surprisingly, despite repeated cycles between poor blood flow at low body temperature and rapid restoration of flow with rewarming during arousal, hibernators do not seem to experience structural damage that jeopardizes renal function (Zancanaro et al. 1999; Jani et al. 2011). This outcome is in sharp contrast to the severe renal damage generally occurred in the nonhibernators, including donated human kidneys which require prolonged cold storage exposure prior to transplantation. Donated human kidneys are cooled down to 4 °C ensuing procuration to preclude the cellular injury from occurring. However, cooling the donor kidneys to be transplanted offers a marginal benefit to transplantation, and storing kidneys from postmortem donors at 4 °C indefinitely sounds far-fetched for now. The risk of rejection of a living-donor kidney transplant increases progressively depending on the increasing duration of cold ischemia, which in turn portends a significant reduction in graft survival (Ojo et al. 1997). In kidney transplantation in nonhibernators including human, ischemia-reperfusion injury is an inevitable relevant consequence that may influence the successful graft outcome (Salvadori et al. 2015). Furthermore, cellular damage resulting from an ischemia-reperfusion injury is a serious obstacle to successful organ transplantation, which has to be surmounted. However, kidneys from the thirteen-lined ground squirrels, which are exposed to several days of cold hypoperfusion followed by warm perfusion, are completely protected against ischemia-reperfusion damage (Jani et al. 2011). Therefore, deeper insight into possible mechanisms by which protection against ischemia-reperfusion damage is achieved during hibernation might be extremely useful for human kidney pathology and transplantation.

In the mammalian kidney, a number of peptides including natriuretic peptides (NPs) are known to play important roles in normal renal physiology and renal system diseases. It is now well established that NPs are implicated in the development of various renal pathologies and protect against cold hypoperfusion and subsequent reperfusion by alleviating the renal ischemia-reperfusion injury (Marumo et al. 1991; Chujo et al. 2008; Cao et al. 2015; Holditch et al. 2017; Rubattu et al. 2019). As a family of peptide hormones with pleiotropic effects, the natriuretic peptide (NP) system consists of NPs [atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP)], the NP receptors (NPR-A, NPR-B, and NPR-C), and the related protease convertases (furin, corin, and proprotein convertase subtilisin/kexin type 6 (PCSK6)). NPs are primarily produced from many cells including cardiomyocytes and are involved in the regulation of cardiovascular- and renal homeostasis and extracellular fluid volume (Woodard and Rosado 2008; Öztop et al. 2018, 2019) and represent relevant protective mechanisms toward the development of renal dysfunctions such as renal failure (Rubattu et al. 2019). NPs elicit multiple discrete biological functions by binding to NPRs. ANP and BNP bind to NPR-A whereas NPR-B is activated by CNP. NPR-A and NPR-B generate the intracellular second messenger cyclic guanosine monophosphate (cGMP). However, all three NPs indiscriminatingly bind to and activate NPR-C, which does not contain the guanylate cyclase (GC) catalytic domain (Nishikimi et al. 2006). The distribution patterns of ANP, BNP and CNP were investigated in kidney tissues of human and various animals (Koseki et al. 1986; Suzuki et al. 1993; Mattingly et al. 1994; Totsune et al. 1994; Herten et al. 1998; Heimeier and Donald 2003; Dong et al. 2016). Among NPs, ANP expression was, however, reported in the frog mesonephric kidney during hibernation in the only study (Fenoglio et al. 2004).

In the light of these studies, it may be proposed the hypothesis that renal NPs may have a role in the protection against cold ischemia/reperfusion insults and renal fibrosis, and that their renal expression may change according to fluctuations in several parameters such as glomerular filtration and renal hemodynamics. To the best of our knowledge, the expression of natriuretic peptides ANP, BNP and CNP had not previously been documented in the kidney of Anatolian ground squirrel (*Spermophilus xanthoprymnus*), a mammalian species that hibernates every winter. Therefore, we sought to delve into the expression and localization patterns of NPs in the pre-hibernating and hibernating Anatolian ground squirrel (*Spermophilus xanthoprymnus*) kidney using immunohistochemistry in an attempt to find out whether hibernation influences NPs expression.

Materials and methods

Animals ethics statement and experimental conditions

Animal experiments were performed in line with the guidelines for Erciyes University Ethics Committee for Animal Care and Use, upon approval (number: 15/140) by Erciyes

University Local Ethics Committee for Animal Experiments (HADYEK). The study material consisted of 12 male Anatolian ground squirrels (*Spermophilus xanthoprymnus*) within the range of of 300-380 g. Animals were wild captured from steppes by Tomahawk Traps (Model #202, Tomahawk, WI, USA) in Develi, Kayseri, Turkey in late August. Therefore, the exact age of the animals is unknown. They were then carried to the laboratory. Animals were quarantined for at least 7 days in cages with a stable ambient temperature of 21 °C under a 12:12 h light-dark cycle until used for experimental purposes so as to make sure they were very healthy. They were then separated into two groups, namely pre-hibernation [control ($n = 6$)] and hibernation (late torpor) [(n = 6) experimental]. Following the anesthetization with intraperitoneal xylazine (11.1 mg/ml) and ketamine (88.9 mg/ml) (Olson and McCabe 1986), pre-hibernating ground squirrels were euthanased with cardiac puncture. Then, the kidney of each animal was quickly excised and dissected. The kidney tissue samples were fixed within 10% formol-alcohol solution, and dehydrated in graded series of ethanol (*70%*, 80%, *96%*, *100%*: 1 h each). After that, the samples were passed through a series of methyl benzoate and benzene, and embedded in paraffin wax for immunohistochemical observation.

Hibernating squirrels were provided with a sunflower seeds, fresh fruit, standard rodent mix, and water *ad libitum* until they stored up ample amounts of fat prior to hibernation. The laboratory temperature was set to 21 ± 1 °C in the beginning and photoperiod of the artificial light-dark cycle (200–0 lx) was set to simulate the outside photoperiod from September 2016 until December 2016. Then, the laboratory temperature was dropped to 6 °C and the foods were taken away from the cages and the lights were switched off completely to ease a natural transition into torpor. In order not to scare torpid squirrels off, a red safe light (3–5 lx) was switched on when going into the laboratory. Three months after first entrance into torpor when squirrels are in hypothermic, full torpid state, the above-mentioned procedures ensued for the experimental group.

Immunohistochemical analysis

Five-micrometer thick sections cut from paraffin-embedded kidney blocks using a rotary microtome (Leica RM2125RT, Nussloch, Germany) were placed on slides pretreated with 3-aminopropyl-ethoxysilane (APES, Sigma–Aldrich, #440140) and overnight kept in an oven at 37 °C. Then, the streptavidin-biotin-peroxidase technique was applied to slides for immunohistochemical staining, as specified before (Özbek et al. 2018). After keeping in the oven at 55 °C for 30 min, slides were dewaxed with xylene and rehydrated by descending series of alcohol concentrations until 70% (100%, 96%,80%, and 70%). After rinsing in phosphate-buffered saline (PBS, Sigma-Aldrich, #P4417) pH 7.4 for 5 min, slides used for immunohistochemical analysis were subjected to antigen retrieval in a citrate buffer (0.01 M, pH 6.0, Thermo Fisher Scientific, #AP-9003-500) for 20 min in a microwave oven at full power. After cooling for 20 min, slides were kept in 0,2% Triton X-100 in PBS for 15 min to improve the permeability. Endogenous peroxidase activity was quenched by subjecting the slides to 3% hydrogen peroxide (H_2O_2) in distilled water in a humidity chamber for 20 min. After that, slides were washed for 15 min in PBS and encircled by a liquid blocker (DakoPen). Ultra V Block (Thermo Fisher Scientific, #TA-125-UB) was applied for 10 min in order to inhibit non-specific binding. After draining excess serum from the slides, sections were overnight incubated with primary antibodies (Table 1) at 4 °C. After washing with PBS, the biotin blocking solution (Biotin Block Part A, #BBA015; Biotin Block Part B, #BBA015) was used in order to block endogenous biotin activity. Routine immunohistochemical procedures were then followed. The staining was visualized with 3-amino-9-ethylcarbazole (AEC, (Thermo Fisher Scientific, #TA-125-HA). After counterstaining with Gill's II hematoxylin, the slides were coverslipped with water-based mounting medium (Thermo Fisher Scientific, # TA-060-UG). Manufacturer's instructions were followed for all dilutions. The specificity of the immunohistochemical staining was validated through positive and negative control sections. Normal rabbit IgG (non-immune sera, Santa Cruz, #sc-2027) was used instead of the primary antibody for negative controls. In addition, the sections were treated with PBS instead of primary antibodies for negative controls. Squirrel atrium was used for positive controls. Immunoreactivity was determined as the presence of red color. Immunohistochemical stainings were repeated at least 3 times for each sample. All the procedures were conducted in a humidity chamber at room temperature unless otherwise specified.

Semi-qualitative evaluation of immunostaining

After the stained sections were examined, photographs were captured using a digital camera (DP74, *Olympus, Tokyo, Japan*) attached to a BX51 microscope (Olympus, Tokyo, Japan). Staining intensity was graded semi-qualitatively as – (negative), ± (very weak staining), + (weak staining), ++ (moderate staining) and +++ (strong staining), as described in our previous study (Öztop et al. 2019). Results of semi-qualitative assessment are presented in Table 2.

Results

For ANP, BNP and CNP positive controls, we used Anatolian ground squirrel atrium in which immunoreactivity was observed as granular reaction for ANP and BNP, and as diffuse staining

Table 1 Primary antibodies used for immunohistochemistry (IHC)

Antibody	Immunogen	Host	Application	Dilution	Catalogue no*	Species reactivity
ANP (FL-153) pAb	Amino acids 1–153 corresponding to full length ANP of human origin	Rabbit	IHC	1/100	sc-20,158	Human, rat, mouse
BNP (FL-134) pAb	Amino acids 27–134 corresponding to C-terminus of BNP of human origin	Rabbit	IHC	1/100	sc-20,159	Human
CNP (H-115) pAb	Amino acids 1–115 corresponding to CNP of human origin	Rabbit	IHC	1/100	sc-20,952	Human, rat, mouse

*All antibodies listed were provided from Santa Cruz Biotechnology

Abbreviations: ANP: Atrial Natriuretic peptide; BNP: Brain Natriuretic peptide; CNP: C-type Natriuretic peptide; pAb: polyclonal antibody

for CNP in atrial cardiomyocytes (Fig. 1, Left Panel) (Öztop et al. 2019). However, any immunostaining was not observed in negative control sections that treat with normal rabbit IgG (non-immune sera) and PBS instead of primary antibodies (Fig. 1, Right Panel). In order to examine NPs expression in pre-hibernating and hibernating squirrel kidneys, we immunostained ANP, BNP and CNP proteins in squirrel kidney sections. ANP, BNP and CNP immunostainings in the kidneys of the pre-hibernating and hibernating squirrels are shown in Figs. 2, 3 and 4. Table 2 presents the semi-quantitative staining intensity of NPs in the renal compartments. Immunohistochemical staining of pre-hibernating and hibernating squirrel kidney tissues demonstrated different distribution patterns of ANP, BNP and CNP in the cortical and medullary regions of kidney. ANP immunoreactivity was weaker than those of BNP and CNP in both periods. In addition, immunoreactivity intensities for all the natriuretic peptides was generally stronger in cortical regions than those in the medullary regions during both periods.

ANP Protein expression in pre-hibernation and hibernation

In the pre-hibernation period, cortical proximal tubules showed weak ANP immunoreactivity. Moderate staining was present at especially the brush border of proximal tubules (Fig. 2A). However, no immunostaining was observed in medullar regions of pre-hibernating animals (Fig. 2 C and E). In the hibernation period, moderate ANP immunoreactivity was seen in cortical proximal tubules although very weak reaction was present in distal tubules (Fig. 2 B). Weak immunostaining was detected in medullary proximal and collecting tubules (Fig. 2D and F). Weak immunostaining for ANP was present in glomeruli in the hibernation period, although very weak staining was detected with ANP in the pre-hibernation period (Fig. 2A and B).

BNP protein expression in pre-hibernation and hibernation

In the pre-hibernation period, some cortical proximal tubules showed strong immunoreactivity to BNP while others had moderate staining. However, some distal tubules stained weakly with BNP whereas others did not (Fig. 3A). Medullary proximal, distal and Henle's loop segments showed very weak BNP immunoreactivity, all of which was in a similar intensity (Fig. 3C and E). In the hibernation period, as in the pre-hibernation, strong immunoreactivity to BNP was observed in some cortical proximal tubules while others exhibited moderate staining. In a similar manner to pre-hibernation, some, not but all, distal tubules were immunostained weakly with BNP (Fig. 3B). Medullary distal, proximal and collecting tubules were immunostained moderately with BNP. Moderate BNP immunoreactivity was seen in Henle's loop segments (Fig. 3D and F). Glomeruli exhibited similar weak immunostaining for BNP in both periods, albeit with a bit more prominent in the hibernation period (Fig. 3A and B).

Table 2 The immunostaining intensities of natriuretic peptides in squirrel kidneys

	Pre-hibernation			Hibernation		
	ANP	BNP	CNP	ANP	BNP	CNP
Glomerulus	±	+	+	+	+	+
Cortical proximal tubules	+	++/+++	+++	++	++/+++	++/+++
Cortical distal tubules	–	+	++	±	+	+
Medullary proximal tubules	–	±	++/+++	+	++	++
Medullary distal tubules	–	±	–	–	++	+
Henle's loop	–	±	++	–	++	++

- negative staining, ±, very weak staining, + weak staining, ++ moderate staining, +++ strong staining

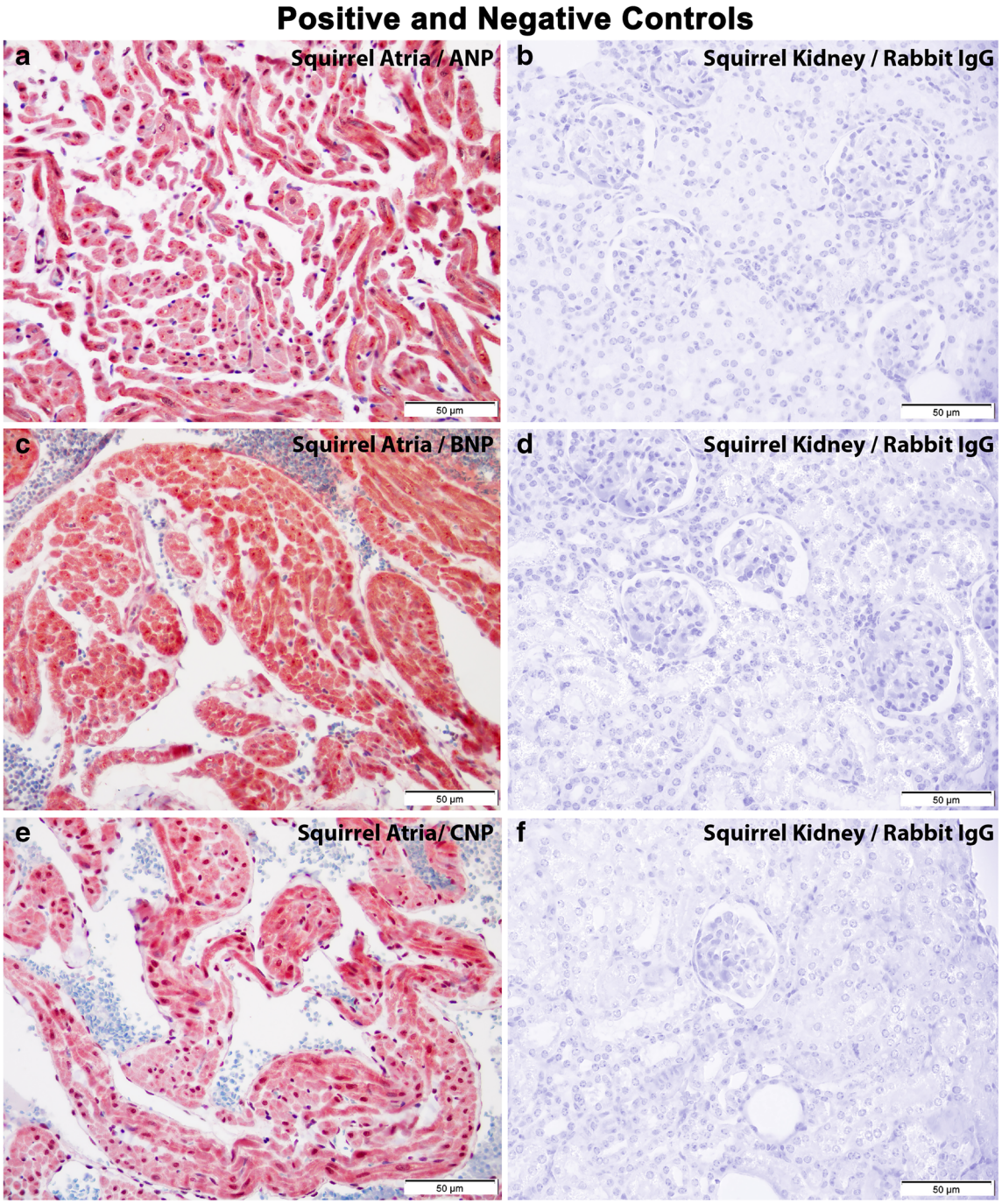


Fig. 1 Representative images of positive and negative control tissues. Positive immunostaining for ANP, BNP and CNP was detected in atrial cardiomyocytes (Fig. 1A, C and E). For negative controls, non-immune rabbit IgG is substituted for the primary antibody and no immunostaining for ANP (Fig. 1B), BNP (Fig. 1D) and CNP (Fig. 1F) was observed in any structure of kidneys. Scale bars: 50 µm

CNP protein expression in pre-hibernation and hibernation

In the pre-hibernation period, most cortical proximal tubules exhibited strong immunoreactivity to CNP. However, distal tubules were immunostained moderately with CNP (Fig. 4A). Medullary proximal tubules exhibited moderate to strong immunoreactivity whereas the Henle's loop segments displayed moderate CNP immunoreactivity, all of which was in a similar intensity (Fig. 4C and E). In the hibernation period, strong immunoreactivity to CNP was observed in some cortical proximal tubules while others exhibited moderate staining. Weak immunostaining was observed in cortical distal tubules (Fig. 4B). Medullary distal and proximal tubules were immunostained weakly and moderately with CNP, respectively. Moderate CNP immunoreactivity was seen in the Henle's

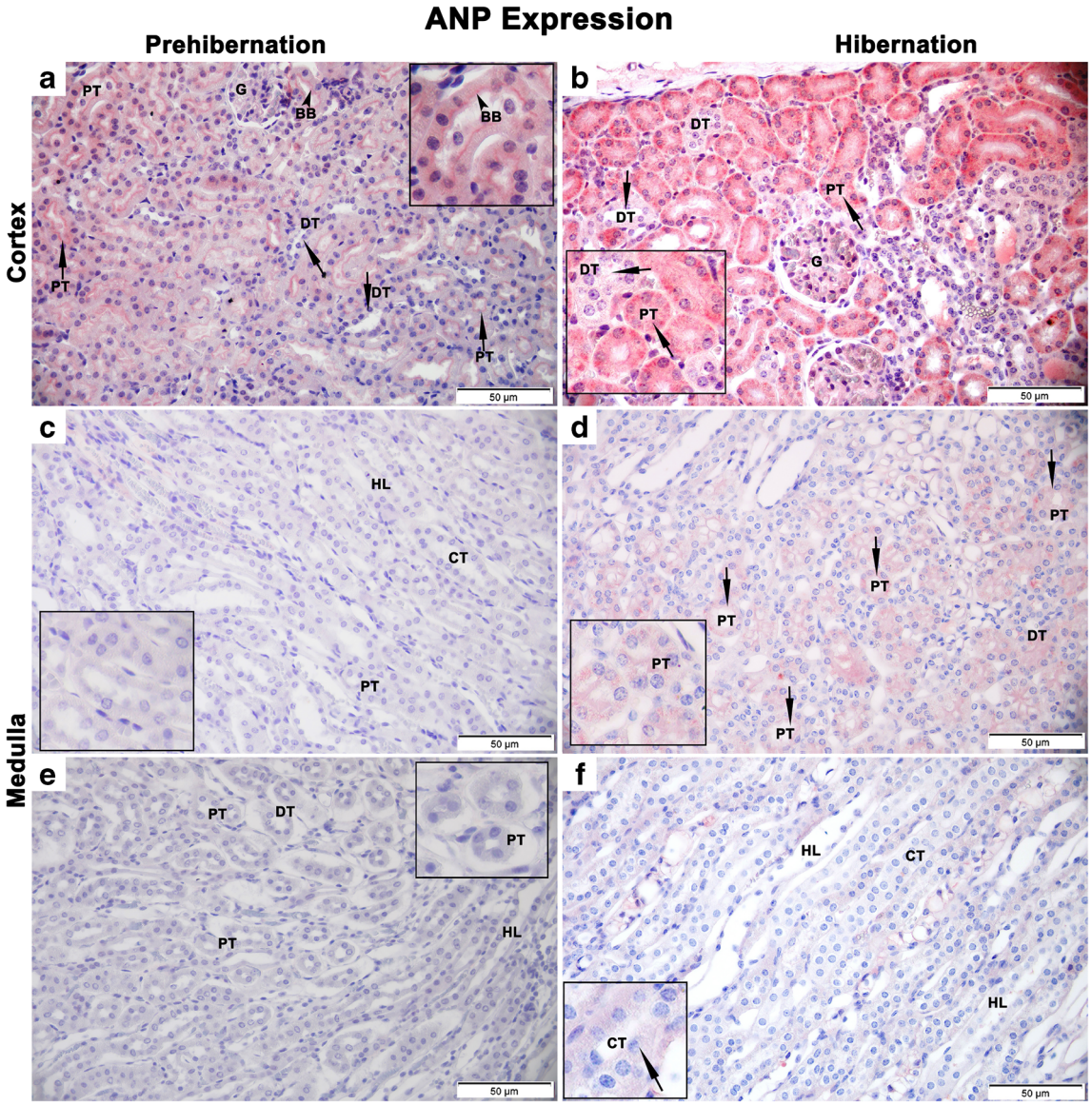


Fig. 2 ANP immunostaining in pre-hibernating and hibernating squirrel kidneys. In the pre-hibernation, cortical proximal tubules (PT) exhibited weak ANP immunoreactivity (arrows) and the staining was at especially the brush border (BB) (arrowheads) (Fig. 2A) although no immunostaining was observed in medullar regions (Fig. 2C and E). In the hibernation period, moderate ANP immunoreactivity (arrows) was seen in cortical proximal tubules (PT) while very weak reaction (arrows) was present in distal tubules (DT) (Fig. 2B). Weak immunostaining (arrows) was present in medullary proximal and collecting tubules (CT) (Fig. 2D and F). Weak immunostaining for ANP was present in glomeruli (G) in the hibernation period, although very weak staining was detected with ANP in the pre-hibernation period (Fig. 2A and B). Scale bars: 50 µm

loop segments. (Fig. 4D and F). Glomeruli exhibited similar weak immunostaining for CNP in both periods, although being a slightly more noticeable in the hibernation period (Fig. 4A and B).

Discussion

This is the first study to explore the distribution patterns of natriuretic peptides in Anatolian ground squirrel kidneys at the pre-hibernating and hibernating periods. Immunohistochemistry has revealed a heterogeneous distribution of NPs in the kidneys. Expression of NPs tended to increase during hibernation. In addition, expressions of BNP and CNP were more noticeable than those of ANP during both periods. The most striking finding is that hibernation might lead to a slight increase in the renal ANP expression. Expression of all the three NPs was more evident in the proximal tubules in both periods. This different expression pattern of NPs may be associated with the kidney structure that seems surprisingly to be well preserved during hibernation, as evidenced by Zancanaro et al. (1999).

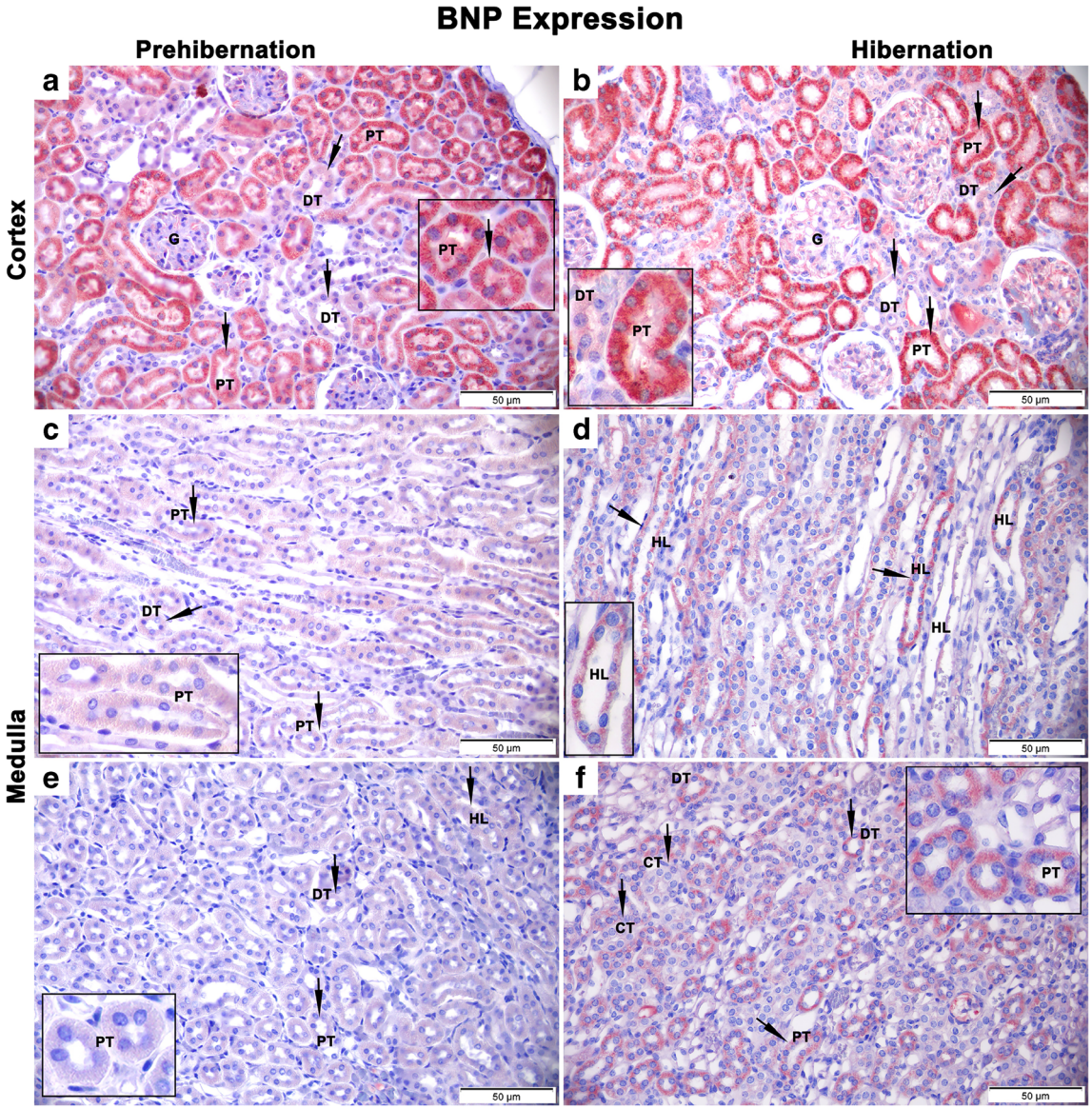


Fig. 3 BNP immunostaining in pre-hibernating and hibernating squirrel kidneys. In the pre-hibernation, cortical proximal tubules (PT) exhibited moderate to strong BNP immunoreactivity (arrows) although weak immunostaining (arrows) was observed in distal tubules (DT) (Fig. 3A). Medullary proximal, distal and Henle's loop (HL) segments showed very weak BNP immunoreactivity (Fig. 3C and E). In the hibernation period, moderate to strong BNP immunoreactivity (arrows) was seen in cortical proximal tubules (PT) while weak reaction (arrows) was present in distal tubules (DT) (Fig. 3B). Moderate immunostaining (arrows) was present in medullary proximal, distal, collecting tubules (CT) and Henle's loop (HL) segments (Fig. 3D and F). Weak BNP immunoreactivity was present in glomeruli (G) in the pre-hibernation and hibernation periods (Fig. 3A and B). Scale bars: 50 µm

ANP of atrial origin is a potent natriuretic, diuretic, antihypertensive and vasodilatory peptide that makes a significant contribution to lowering blood pressure (de Bold 1979). It dilates the afferent arterioles and constricts the efferent arterioles in the kidney, which in turn increases renal vasodilation and glomerular filtration rate (Lanese et al. 1991). ANP also regulates redistribution of renal medullary blood flow (Davis and Briggs 1987). There is growing evidence that ANP can reduce ischemia-reperfusion damage. Shaw et al. (1987) and Nakamoto et al. (1987) reported that infusion of ANP after renal ischemia could improve glomerular filtration rate, and prevent renal tissue damage by returning the histological appearance of kidneys to normal. In addition, Marumo et al. (1991) showed that human ANP (hANP) could prolong the preservation duration of dog kidneys. Chujo et al. (2008, 2010) showed that ANP attenuates I/R-induced acute renal injury. These experimental reports suggest that ANP is reno-protective against the renal injury caused by ischemia-reperfusion insults. In fact, the available literature and our results showing slightly increased expression of ANP protein in the hypothermia support the possibility that ANP may be a clinically promising candidate for storing the kidneys for the kidney transplantation.

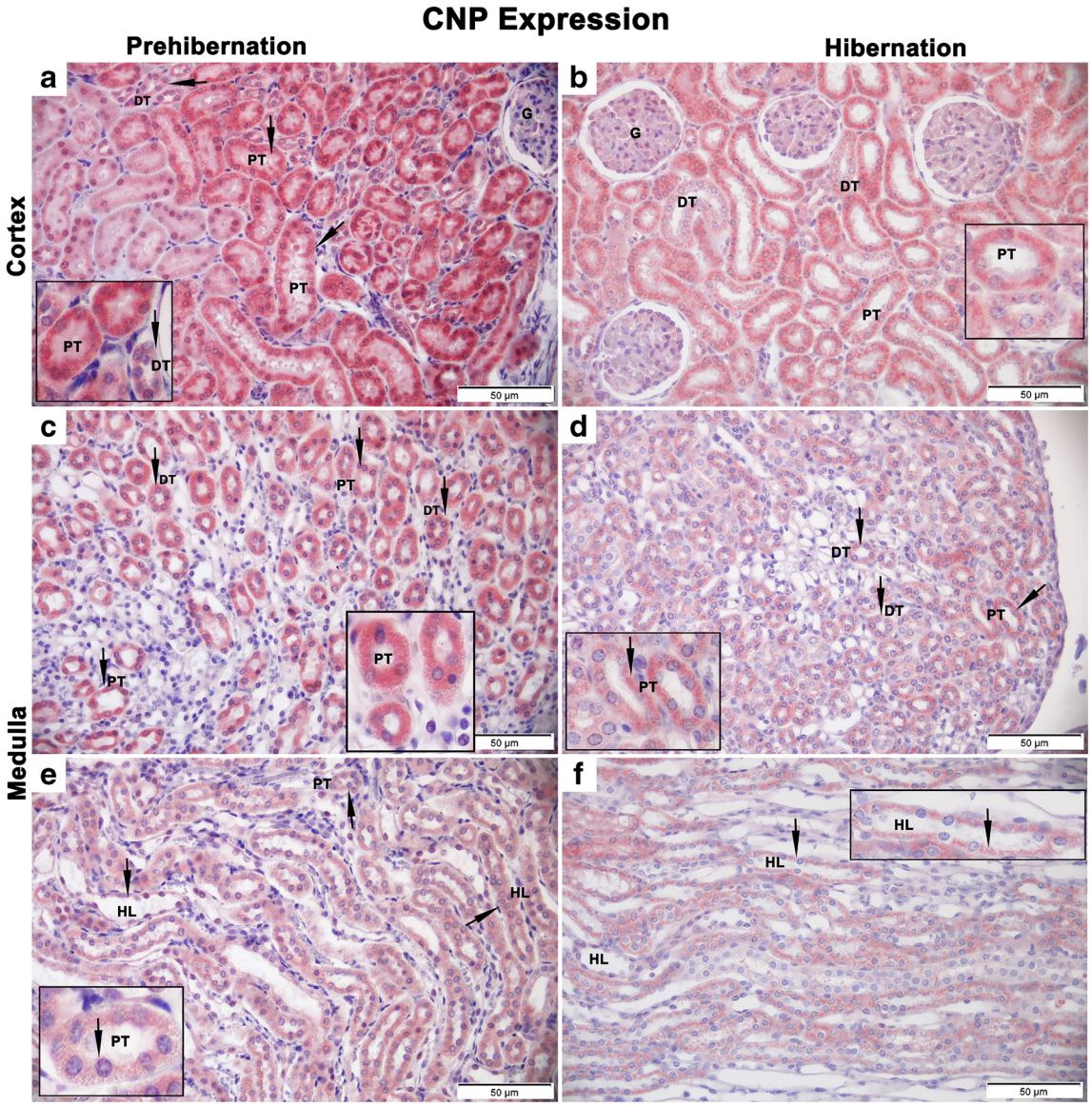


Fig. 4 CNP immunostaining in pre-hibernating and hibernating squirrel kidneys. In the pre-hibernation, cortical proximal tubules (PT) exhibited strong CNP immunoreactivity (arrows) although moderate immunostaining (arrows) was detected in distal tubules (DT) (Fig. 4A). Medullary proximal tubules (PT) showed moderate to strong CNP immunoreactivity whereas Henle's loop (HL) segments showed moderate immunoreactivity (Fig. 4C and E). In the hibernation period, moderate to strong CNP immunoreactivity (arrows) was seen in cortical proximal tubules (PT) while weak reaction (arrows) was seen in distal tubules (DT) (Fig. 4B). Moderate and strong immunostaining (arrows) were present in medullary distal and proximal tubules, respectively. Henle's loop (HL) segments had moderate CNP immunoreactivity (Fig. 4D and F). Glomeruli (G) exhibited similar weak immunostaining for CNP in both periods, albeit a slightly more noticeable in the hibernation period (Fig. 4A and B). Scale bars: 50 µm

The previous studies have provided ample evidence regarding ANP distribution in the kidneys of various mammalian species using immunohistochemistry, radioisotopes and *in situ* hybridization methods (Koseki et al. 1986; Mckenzie et al. 1991; Ramirez et al. 1992; Saba et al. 1993; Totsune et al. 1994; Lai et al. 2002; Fenoglio et al. 2004; Dong et al. 2016). Of those studies, Koseki et al. (1986) showed that ANP localizes in renal arteries, glomeruli and inner medullary collecting tubules using autoradiographic method. Mckenzie et al. (1991) found that ANP was localized specially to the outer medullary and cortical collecting tubules and ducts, and to the proximal convoluted tubule brush border of human, monkey, pig, mouse, and rat kidneys. A similar distribution of ANP was found in rat (Ramirez et al. 1992) and human (Saba et al. 1993; Totsune et al. 1994) renal components, with a stronger staining in the rat proximal convoluted tubules. In a study by Lai et al. (2002), ANP and its mRNA were detected in the proximal, distal and collecting tubules of normal and diabetic rat kidneys using *in situ* hybridization and immunohistochemistry. The level of ANP mRNA and its

peptide increased significantly in the renal tissue of diabetic rats compared to normal rats, indicating that ANP may be implicated in the renal adaptations and functions in diabetic conditions. Dong et al. (2016) found prominent pro-ANP/ANP immunoreactivity in proximal and distal convoluted tubules, collecting ducts and thick ascending limbs although weak staining was present in thin limbs and glomeruli. In addition to these, Fenoglio et al. (2004) found that ANP immunoreactivity was similar in active and hibernating frog mesonephros, albeit tending to slightly decrease in the hibernating frogs. We used antibody detected all the forms of ANP. Our pre-hibernating findings dovetail with those reported by the available studies. However, our hibernating findings are in contrast with those of Fenoglio et al. (2004). Therefore, both the present findings and previous findings of ANP in the renal segments strongly support that ANP may be produced by kidneys, thereby regulating the renal homoeostasis in an autocrine manner. Moreover, slightly increased expression of ANP in our study could contribute to optimization of renal function during hibernation. Some more ANP immunoreactivity in the hibernation than pre-hibernation might also represent a stored form of ANP ready for use that is probably released in response to appropriate stimuli when squirrels arouse. It is now well established that hibernation is linked with greater resistance to ischemia-reperfusion injury including suppression of inflammatory responses (Lindell et al. 2005; Kurtz et al. 2006). Therefore, owing to its anti-inflammatory and anti-fibrotic properties (Mukaddam-Daher 2006), ANP expression tending to slightly increase in hibernating squirrel kidneys might provide higher resistance to ischemia-reperfusion damage and its associated inflammation and fibrosis that would otherwise occur in nonhibernators. Moreover, the increased expression in the hibernation in response to cold conditions may compensate for changes in renal hemodynamics and body fluid balance. However, this merits further experimental investigations into how ANP is involved in these processes.

BNP acts principally on the kidneys with physiological effects similar to those of ANP, stimulating tubular diuresis and natriuresis (Koga et al. 2012). Several lines of evidence have shown putative protective effects of BNP on various renal dysfunctions and diseases (Suganami et al. 2001; Makino et al. 2006; Kim et al. 2010; Cao et al. 2015; Holditch et al. 2017). Of note, Makino et al. (2006) observed that chronic expression of BNP prevents the kidney from developing a diabetic renal injury in mice. Kim et al. (2010) showed that BNP may provide a short-term protection against only ischemia for up to 45 min followed by 3 h reperfusion in rats because long-term ischemia (for >45 min) had irreversible loss of renal function. Another study showed that BNP mitigates renal tubular damage caused by ischemia for 30 min followed by 12 and 24 h reperfusion in mice (Cao et al. 2015). Based on the above-mentioned findings, it may assert that BNP has a reno-protective effect on ischemia-reperfusion injury at short-term intervals. On the other hand, some studies have reported the existence and expression of BNP in kidneys. Marumo et al. (1992) demonstrated the existence of BNP in the porcine kidney. Totsune et al. (1994) showed positive BNP immunostaining in only the distal tubular segments of human kidney. Nakagawa et al. (1995) showed the presence of BNP-like immunoreactivity in the mouse kidney. Herten et al. (1998) found BNP immunoreactivity mainly localized to distal tubular segments of human kidney. Consistent with the literature available, we observed BNP immunoreactivity in other renal components, as well as distal tubules, during both pre-hibernating and hibernating squirrel kidneys. Intensity of immunoreactive BNP tended to increase during hibernation, suggesting that hibernation could affect BNP expression. Therefore, a tendency to increase the expression of BNP in hibernating kidneys might be a protective role in ischemia/reperfusion injury that would otherwise occur in nonhibernators. Taken together, these results suggest that BNP does have a protective effect on murine IRI and may be a promising candidate for the treatment of renal ischemia-reperfusion injury and other renal diseases in clinical practices. However, it should be cautious about interpreting these results and extrapolating them to clinical situations. Therefore, our study and the current literature would open up the possibility that BNP through its renoprotective effects might be therapeutically relevant to the prevention of renal diseases.

CNP regulates several functions such as vascular tone and salt excretion (Surendran and Simon 2003). Although initially detected in pig brain (Sudoh et al. 1990), CNP mRNA and protein expression have been detected in other organs such as heart and kidneys (Komatsu et al. 1991; Stingo et al. 1992; Suga et al. 1992; Vollmar et al. 1993). Many researchers have showed that CNP mRNA and protein are detectable in the human (Komatsu et al., 1991; Dean et al. 1994; Mattingly et al. 1994; Totsune et al. 1994), mouse (Dean et al. 1994) rat (Komatsu et al. 1991; Suzuki et al. 1993; Dean et al. 1994; Terada et al. 1994), dog (Nir et al. 1994), and Spinifex Hopping mouse, *Notomys alexis* (Heimeier and Donald 2003) kidney and in cultured human (Komatsu et al. 1991; Ueda et al. 1991; Dean et al. 1994; Mattingly et al. 1994; Nir et al. 1994), opossum (Nir et al. 1994) and rat (Suzuki et al. 1993) kidney cells. These findings show that CNP is expressed locally by the kidneys. Consistent with those mentioned above, our findings revealed the presence of CNP protein in various renal segments of squirrel kidney using immunohistochemistry. All these studies show that CNP, considered to be expressed locally, plays an important autocrine or paracrine role through its receptor, NPR-B, in the mammalian kidney in order to maintain the renal hemodynamics and homeostasis. In consideration of the present data, the expression of CNP protein that remained almost unchanged in hypothermia when compared to euthermia might be important for squirrel kidneys to adapt to cold conditions of hibernation.

To conclude, this study firstly delves into the expression of NPs in Anatolian ground squirrel kidneys. Expressions of BNP and CNP were more noticeable than those of ANP during both periods. The present results demonstrate that expression of NPs tended to increase during hibernation, suggesting that NPs are expressed by the squirrel kidney and form a renal natriuretic peptide system that may be involved in the renoprotective effects against ischemia-reperfusion insults. We reason that different expression pattern of NPs may contribute to the renal adaptation and well-preserved renal structure during hibernation. A deeper insight into how kidneys are protected during hibernation may help us understand the underlying pathophysiological mechanisms occurring during renal transplantation. Therefore, further experimental studies are needed to understand mechanistically how NPs affect renal function during hibernation and to better understand the functional importance and protective roles of NPs in kidneys during hibernation.

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Compliance with ethical standards

Statement of animal rights The procedure involving all the animal experiments was conducted according to the guidelines of Erciyes University Animal Care and Use Committee.

Conflict of interest The authors declare that there is no conflict of interest.

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